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Stromelysin-3 (ST-3), a member of the matrix metalloproteinase (MMP) family, is expressed in stromal cells surrounding invasive breast carcinoma cells where it undergoes processing to its active form by an intracellular, proprotein convertase-dependent pathway. While strong correlations between disease progression and ST-3 expression suggest that the proteinase plays a critical role in breast cancer, human ST-3 is the only matrix metalloproteinase that does not express a marked ability to degrade components of the extracellular matrix (ECM). To further characterize the role of ST-3 *in vivo*, our attention focused on the analysis of lines of transgenic mice wherein the whey acidic protein (WAP) gene was used to direct human ST-3 expression to mammary tissue during late pregnancy through lactation. In these studies, high levels of the ST-3 transgene were generated *in vivo* which unexpectedly triggered a massive involution program. Surprisingly, ST-3 not only induced an apoptotic program, but also initiated changes in the underlying basement membrane *in vivo*. In addition, ST-3 expression also appeared to induce an exaggerated angiogenic response in involuting tissues. These data provide the first evidence that ST-3 can regulate mammary epithelial-ECM interactions and angiogenesis *vivo*.

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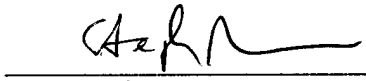

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I. INTRODUCTION

Current evidence suggests that breast carcinoma cells invade local tissues and metastasize by either expressing, or inducing the expression of, proteolytic enzymes that degrade structural barriers established by the extracellular matrix (ECM)[1-3]. Although the identity of the specific proteinases that lend cancer cells their invasive potential remains the subject of conjecture, attention has recently focused on the matrix-degrading metalloproteinases (MMPs) - a family of at least 14 zinc-dependent proteolytic enzymes whose overlapping substrate specificities include all of the major components of the ECM[1-5]. Consistent with their presumed role in tumor progression, *in situ* analyses of a variety of cancerous tissues have confirmed heightened levels of expression of one or more MMPs in tumor and/or surrounding stromal tissues[6-8]. Furthermore, a range of *in vitro* as well as *in vivo* studies have demonstrated that invasion and metastasis can be affected by altering MMP activity[9-12]. Given these findings, efforts have intensified to identify those MMPs that might be used as diagnostic indicators or potential targets for pharmacologic intervention in breast as well as other cancers.

Until recently, attempts to implicate specific MMPs in breast cancer progression were based on the assumption that all of the major proteinases belonging to this gene family had been identified and characterized[2]. Unexpectedly however, differential screens of breast cancer cDNA libraries led to the tentative identification of a new member of the MMP family, termed stromelysin-3, on the basis of its apparent homology to stromelysin-1 and -2 (two other members of this gene family that had been previously characterized)[13]. The expressed gene product was predicted to encode a 488-residue protein containing i) a candidate leader sequence, ii) a highly conserved PRCGXPD motif believed to maintain the latency of MMP zymogens, iii) a zinc-binding catalytic motif and iv) a carboxyl-terminal domain with sequence homology to the heme-binding protein, hemopexin and the ECM molecule, vitronectin[13]. Interestingly, in early studies all invasive breast carcinomas examined thus far, ST-3 was not a product of the neoplastic cells themselves, but rather the surrounding stromal cells[6,12,13]. However, more recent analyses have demonstrated that tumor cells themselves can express ST-3 as well[14].

Given the structural similarity between ST-3 and other members of the MMP family, it was postulated that the enzyme would be secreted as a zymogen whose extracellular activation at the tumor-stroma interface would follow the destabilization of the ligand formed between the Cys in the PRCGVPD domain and the Zn^{+2} in the catalytic domain[6,12,13]. In a scheme analogous to that established for the other MMPs, ST-3 could then under autoproteolytic processing to a mature, active form which presumably would catalyze the degradation of critical ECM components localized in either the basement membrane or stroma[4,5]. However, despite the structural similarities that exist between ST-3 and other MMP members, additional studies indicate that i) the primary sequence of ST-3 is distinct from all other members of the MMP family and ii) the assumed role of ST-3 in ECM remodeling cannot be readily confirmed[4,15]. First, comparisons of the catalytic domains of the MMPs suggest that ST-3 belongs in a new subgroup relative to all other members of this gene family and that its closest homology lies with the bacterial metalloproteinases[4]. Consistent with the contention that ST-3 is a structurally distinct entity in the MMP family, the human ST-3 gene has been localized to the long arm of chromosome 22, a position which differs from those of all other MMP genes[4,12]. Second, although the expression of the human ST-3 protein had not yet been reported (this was later determined by our group; see below), the homologous mouse enzyme (~80% homology identity at the amino acid level) could only be isolated in a truncated form that expressed weak proteolytic activity[15]. Indeed, these results led Murphy and colleagues to conclude that "the evidence that mature full length stromelysin-3 is a metalloproteinase could not be substantiated and the precise role of this protein

in vivo remained to be elucidated."[15] Taken together, these findings indicated that despite the provocative correlative link established between ST-3 expression and breast cancer progression, the role of this proteinase in invasive events remained undefined. In this proposal, we sought to use a series of *in vitro* as well as *in vivo* biochemical and molecular approaches to i)purify and characterize ST-3 expressed by stably-transfected mammalian cells, ii) determine the mechanism by which the ST-3 zymogen is activated and regulated, iii) determine the ability of stromelysin-3 to regulate the invasive potential of breast carcinoma cells *in vitro* and iv) characterize the role of ST-3 *in vivo* in a transgenic mouse model. To date, the first three aims have been completed and our efforts have now focused on characterizing the role of ST-3 *in vivo* in a transgenic mouse model generated in our laboratories.

II. BODY

During the previous granting periods, we completed our analysis of the ST-3/proprotein convertase axis, demonstrated that the processing motif encrypted in ST-3 may also operate in the related membrane-type matrix metalloproteinases (i.e., MT1, 2, 3, 4-MMP), and characterized the inability of ST-3-transfected cells to express an invasive phenotype *in vitro* [16-18]. While the first two findings clearly indicated that the proprotein convertases play a critical role in regulating the activities of multiple matrix metalloproteinases, the inability of active ST-3 to degrade extracellular matrix components or promote invasive behavior precluded any predictions of the enzyme's relevance to tumor progression *in vivo*. However, in our last progress report, we indicated that in preliminary analyses of one line of ST-3 transgenic mice, an unusual phenotype was observed wherein the normal pattern of lobuloalveolar development that occurs during pregnancy was disrupted. We now report our findings following the screening of eight independent transgenic lines established in our laboratory. These studies have led to i) the description of an ST-3-triggered premature involution program involving mammary epithelial cell apoptosis and basement membrane remodeling, ii) an unanticipated association between ST-3 expression and angiogenesis and iii) the development of a serum-free mammary explant model for utilizing transgenic tissues to determine the expression, processing and functional activity of ST-3 *in vitro*.

ST-3-INDUCED MAMMARY GLAND INVOLUTION

As indicated earlier, preliminary attempts to target transgene expression specifically to the stroma using an RSV promoter were unsuccessful (see prior Progress Report). As an alternative, we opted to place ST-3 expression under the control of regulatory elements of the mammary epithelial cell-specific, pregnancy -responsive whey acidic protein (WAP) gene[19,20]. Because ST-3 is normally expressed during mammary gland involution (a state that closely parallels many of the matrix-remodeling events observed in carcinomatous states[21,22]), our rationale was to express the transgene during mid-pregnancy and through lactation in order to observe the effect exerted on the well-characterized mammary gland phenotype. (It should be noted that a similar approach has been used by other groups to characterize the role of stromelysin-1 in mammary gland pathophysiology[19,23]. However, despite the similarity of their names, stromelysin-1 and -3 are not closely related gene products and display completely different mechanisms of regulation and activity[16,17]). Thus, transgenic mice were generated using a chimeric recombinant DNA composed of the WAP promoter and the entire ST-3 coding region. The WAP promoter was a 2.6-kb DNA corresponding to the 5' end of the WAP gene extending into the first exon just 5' of the first AUG as described[19,20]. The WAP-ST-3 construct was then injected into 300 embryos and implanted into 10 mice. Following a screening and breeding plan similar to that described previously for the

RSV-ST-3 construct, eight positive mice were obtained with the transgene integrated into the founder germlines (Fig. 1). When tissues from transgenic mice were examined by RT-PCR and Northern blot analysis (data not shown), ST-3 was found to be highly expressed in three lines (designated herein as P1, P3 and P9). As described previously, ST-3 expression was limited to the mammary gland (low levels were found in the brain of only one founder; data not shown) wherein expression was readily detected from mid-pregnancy through lactation (Fig. 2; wild-type animals express only low levels of ST-3 during involution as previously described[21]). Prior to performing histologic analyses of transgenic mice mammary glands, we noted a heightened level of pup death in several transgenic lines wherein the newborns could be rescued by foster mothers (e.g., in the P1 line, only 53 ±13% of the pups survived compared to 80 ±8% of the control mice in first litters; n=16). Histologic analyses of transgenic glands demonstrated only subtle changes prior to lactation (i.e., in virgin or mid-pregnant glands; Figs. 3 and 4), while the transgenic glands appeared to have undergone a premature involution program during early lactation (Fig. 5). While mid-pregnant glands appeared grossly normal, closer examination by histochemical and electron microscopic analyses demonstrated that apoptotic cell number increased significantly as early as day 16 of pregnancy (as determined by a terminal deoxynucleotidyl transferase-based assay; Fig. 6). Given the fact that ST-3-transfected epithelial cells do not display apoptotic changes *in vitro* (data not shown), we reconsidered the possibility that ST-3 may mediate apoptosis by directly affecting basement membrane structure/function *in vivo* (which consequently could trigger apoptosis)[24,25]. This possibility was further suggested by recent findings that human ST-3 has been localized to basement membranes surrounding carcinomatous sites *in vivo*.[14] Indeed, mid-pregnant as well as lactating glands from transgenic mice displayed marked changes in basement membrane structure (Fig. 6, 7). Despite these changes, normal and transgenic tissues appeared similar when involution was purposefully induced by weaning (Fig. 8). While changes in basement membrane-associated laminin, type VI collagen, entactin or heparan sulfate proteoglycan are only now being examined, these data provide the first evidence that ST-3 can directly or indirectly alter basement membrane structure as well as mammary epithelial cell function *in vivo*. Analyses of i) ST-3 distribution at the protein level, ii) WAP, lactalbumin and β-casein gene expression and iii) involution markers (stromelysin-1, MT1-MMP, SGP-2, etc.) are also underway.

A POTENTIAL ROLE FOR ST-3 IN ANGIOGENESIS

While examining whole mounts of lactating transgenic mice, we noted a marked increase in gross vascularity (data not shown). Currently, we are unable to determine whether ST-3 directly stimulates angiogenesis or alternatively, induces an angiogenic response secondary to the initiation of a premature involution program during lactation. Because a role for ST-3 in angiogenesis has not been previously predicted, we sought to determine whether the proteinase is normally expressed during neovascularization. To this end, we constructed an *in vitro* model of angiogenesis wherein mouse tissue explants (1 mm^3) were embedded in 3-dimensional collagen gels and cultured in Transwells according to a modification of the technique developed by Nicosia and colleagues[26]. As shown in Figure 9, neovessels are generated during a two week incubation period wherein a complex pattern of anastomosing vessels are formed. Ultrastructural studies have demonstrated that the neovessels are surrounded by pericytes, linked by tight junctions and invested with a basement membrane (Fig. 9). Interestingly, coincident with the expression of the angiogenic phenotype, high levels of ST-3 were detected at both the mRNA and protein level (data not shown). These data provide the first demonstration that ST-3 expression is linked to angiogenic states. To determine the role of ST-3 in neovessel formation, experiments are

underway wherein explants are co-cultured either with ST-3 transfected cells or with ST-3 antisense oligonucleotides[27].

IN VITRO EXPLANT MODEL OF TRANSGENIC MAMMARY GLANDS

While these data provide some of the first insights into ST-3 function *in vivo*, further mechanistic insights are hindered by the limitations inherent in whole animal experiments. For example, to directly and specifically implicate enzymically active ST-3 in the involution program described above, ST-3 transgenic animals would have to be "rescued" by breeding with mice carrying the TIMP-1 transgene[28] (TIMP or the tissue inhibitor of metalloproteinases is an endogenous inhibitor of ST-3[16,17]). Alternatively, if the mammary involution program could be triggered *in vitro*, the experiments could be readily performed by simply culturing mammary explants with recombinant TIMP-1. Similarly, changes in ST-3 expression and processing, basement membrane turnover, milk protein synthesis, etc. could all be evaluated directly if the *in vivo* event could be recapitulated *in vitro*. To this end, we initially sought to develop the whole gland mammary explant model originally described by Nandi et al[29]. While we were able to reproduce these results, the explant model i) requires the use of very high O_2 concentrations (50-95%) which might perturb key developmental programs and ii) is not easily accessed for biochemical analyses since the intact gland is utilized. Alternatively, recent studies have demonstrated that explants of primed (progesterone/estradiol) or pregnant mice can be cultured *in vitro* and be induced to undergo or maintain, respectively, lobuloalveolar differentiation under serum-free conditions *in vitro*[30]. In brief, the fourth (upper abdominal) mammary glands of hormonally primed normal/transgenic virgin mice or pregnant normal/transgenic mice are removed, placed in Hepes-buffered MEM and sterilely cut into 1-2 mm fragments. Approximately 20 explants are obtained from one abdominal gland of a primed virgin mouse (versus ~200 explants from the combined mammary glands of one pregnant mouse). The tissues (~20 explants) are then placed into plastic flasks and rotated as described[30]. After seeding the explants, the tissues are held in MEM199 in the presence of aldosterone, prolactin, insulin and cortisol[30]. Under these conditions, explants undergo lobuloalveolar differentiation and synthesize milk proteins (pregnant glands maintain their differentiated state). While further studies are underway to determine whether the involution program develops in explants from transgenic glands, tissues from normal mice can be induced to involute by simply withdrawing prolactin and cortisol. This model will now be used to directly assess the impact of the effect of ST-3 expression on mammary gland development and function *in vitro*.

III. CONCLUSION

The research completed to date have allowed us to demonstrate that although human ST-3 expresses only limited proteolytic activity *in vitro*, its *in vivo* expression initiates a complex apoptosis/involution a massive program and perhaps, angiogenesis as well. These data provide some of the first insights into ST-3 function *in vivo* and strongly suggest that the proteinase may play an important role in regulating tumor progression by simultaneously regulating matrix turnover and angiogenesis. While normal epithelial cells undergo apoptosis under these conditions, these events would allow neoplastic cells (which resist apoptotic signals[31]) to invade and survive in the surrounding tissues.

IV. REFERENCES

1. Laiho, M., Keski-Oja, J. Growth factors in the regulation of pericellular proteolysis: A review. Cancer Res. 49:2533-2553, 1989.

2. MacDonald, N.J.and Steeg, P.S. Molecular basis of tumour metastasis. In Cancer Surveys, The Molecular Pathology of Cancer, 16:175-199, 1993.

3. Liotta, L.A., Steeg, P.S., and Stetler-Stevenson, W.G. Cancer metastasis and angiogenesis: An imbalance of positive and negative regulation. Cell 64:327-336, 1991.

4. Matrisian, L.M. The matrix-degrading metalloproteinases. BioEssays 14:455-463, 1992.

5. Woessner, J.F. Jr. Matrix metalloproteinases and their inhibitors in connective tissue remodeling. FASEB J. 5:2145-2154, 1991.

6. Wolf, C., Rouyer, N., Lutz, Y., Adida, C., Loriot, M., Bellocq, J.P., Chambon, P. and Basset, P. Stromelysin 3 belongs to a subgroup of proteinases expressed in breast carcinoma fibroblastic cells and possibly implicated in tumor progression. Proc. Natl. Acad. Sci. USA 90:1843-1847, 1993.

7. Monteagudo, C., Merino, M.J., San-Juan, J., Liotta, L.A., Stetler-Stevenson, W.G. Immunohistochemical distribution of type IV collagenase in normal, benign, and malignant breast tissue. Am J Pathol 136:585-592, 1990.

8. Polette, M., Clavel, C., Cockett, M., de Bentzmann, S.G., Murphy, G.and Birembaut, P. Detection and localization of mRNAs encoding matrix metalloproteinases and their tissue inhibitor in human breast pathology. Invasion Metastasis 13:31-37, 1993.

9. Mignatti, P., Robbins, E., and Rifkin, D.B. Tumor invasion through the human amniotic membrane: requirement for a proteinase cascade. Cell 47:487-498, 1986.

10. DeClerck, Y.A., Perez, N., Shimada, H., Boone, T.C., Langley, K.E., and Taylor, S.M. Inhibition of invasion and metastasis in cells transfected with an inhibitor of metalloproteinases. Cancer Res. 52:701-708, 1992.

11. Powell, W.C., Knox, J.D., Navre, M., Grogan, T.M., Kittelson, J., Nagle, R.B., and Bowden, G.T. Expression of the metalloproteinase matrilysin in DU-145 cells increases their invasive potential in severe combined immunodeficient mice. Cancer Res. 53:417-422, 1993.

12. Basset, P., Wolf, C. and Chambon, P. Expression of the stromelysin-3 gene in fibroblastic cells of invasive carcinomas of the breast and other human tissues: a review. Breast Cancer Res. Treatment 24:185-193, 1993.

13. Basset, P., Bellocq, J.P., Wolf, C., Stoll, I., Hutin, P., Limacher, J.M., Podhajcer, O.L., Chenard, M.P., Rio, M.C., and Chambon, P. A novel metalloproteinase gene specifically expressed in stromal cells of breast carcinomas. Nature 348:699-704, 1990.

14. Bolon, I., Devouassoux, M., Robert, C., Moro, D., Brambilla, C., and Brambilla, E. Expression of urokinase-type plasminogen activator, stromelysin 1, stromelysin 3, and matrillysin genes in lung carcinomas. Am. J. Pathol. 150:1619-1629, 1997.

15. Murphy, G., Segain, J.P., O'Shea, M., Cockett, M., Ioannou, C., Lefebvre, O., Chambon, P., and Basset, P. The 28-kDa N-terminal domain of mouse stromelysin-3 has the general properties of a weak metalloproteinase. J. Biol. Chem. 268:15435-15441, 1993.

16. Pei, D., Majmudar, G. and Weiss, S.J. Hydrolytic inactivation of a breast carcinoma cell-derived serpin by human stromelysin-3. J. Biol. Chem. 269:25849-25855, 1994.

17. Pei, D. and Weiss, S. Furin-dependent intracellular activation of the human stromelysin-3 zymogen. Nature 375:244-247, 1995.

18. Pei, D., and Weiss, S.J. Transmembrane-deletion mutants of the membrane-type matrix metalloproteinase-1 activate progelatinase A and express intrinsic matrix-degrading activity. J. Biol. Chem. 271:9135-9140, 1996.

19. Sympson, C.J., Talhouk, R.S., Alexander, C.M., Chin, J.R., Clift, S.M., Bissell, M.J., and Werb, Z. Targeted expression of stromelysin-1 in mammary gland provides evidence for a role of proteinases in branching morphogenesis and the requirement for an intact basement membrane for tissue-specific gene expression. J. Cell Biol. 125:681-693, 1994.

20. Gallahan, D., Jhappan, C., Robinson, G., Henninghausen, L., Sharp, R., Kordon, E., Callahan, R., Merlino, G., and Smith, G.H. Expression of a truncated int3 gene in developing secretory mammary epithelium specifically retards lobular differentiation resulting in tumorigenesis. Cancer Res. 56:1775-1785, 1996.

21. Lefebvre, O., Wolf, C., Limacher, J.M., Hutin, P., Wendling, C., LeMeur, M., Basset, P., and Rio, M.C. The breast cancer-associated stromelysin-3 gene is expressed during mouse mammary gland apoptosis. J. Cell Biol. 119:997-1002, 1992.

22. Li, F., Strange, R., Friis, R.R., Djonov, V., Altermatt, H.J., Saurer, S., Niemann, H., and Andres, A.C. Expression of stromelysin-1 and TIMP-1 in the involuting mammary gland and in early invasive tumors of the mouse. Int. J. Cancer 59:560-568, 1994.

23. Witty, J.P., Wright, J.H., and Matrisian, L.M. Matrix metalloproteinasas are expressed during ductal and alveolar mammary morphogenesis, and misregulation of stromelysin-1 transgenic mice induces unscheduled alveolar development. Mol. Biol. Cell 6:1287-1303, 1995.

24. Ashkenas, J., and Werb, Z. Proteolysis and the biochemistry of life-or-death decisions (Commentary). J. Exp. Med. 183:1947-1951, 1996.

25. Lund, L.R., Romer, J., Thomasset, N., Solberg, H., Pyke, C., Bissell, M.J., Dano, K., and Werb, Z. Two distinct phases of apoptosis in mammary gland involution: proteinase-independent and -dependent pathways. Development (Camb). 122:181-193, 1996.

26. Nicosia, R.F., and Tszynski, G.P. Matrix-bound thrombospondin promotes angiogenesis *in vitro*. J. Cell Biol. 124:183-193, 1994.

27. Lochter, A., Srebrow, A., Sympson, C.J., Terracio. N., Werb, Z., and Bissell, M.J. Misregulation of stromelysin-1 expression in mouse mammary tumor cells accompanies acquisition of stromelysin-1-dependent invasive properties. J. Biol. Chem. 272:5007-5015, 1997.

28. Alexander, C.M., Howard, E.W., Bissell, M.J., and Werb, Z. Rescue of mammary epithelial cell apoptosis and entactin degradation by a tissue inhibitor of metalloproteinases-1 transgene. J. Cell Biol. 135:1669-1677, 1996.

29. Ichinose, R.R., Nandi, S. Influence of hormones on lobuloalveolar differentiation of mouse mammary glands *in vivo*. J. Endocrinol. 35:331-340, 1966.

30. Robinson, S.D., Roberts, A.B., and Daniel. C.W. TGFβ suppresses casein synthesis in mouse mammary explants and may play a role in controlling milk levels during pregnancy. J. Cell Biol. 120:245-251, 1993.

31. Frisch, S.M., Vuori, K., Ruoslahti, E., and Chan-Hui, P.Y. Control of adhesion-dependent cell survival by focal adhesion kinase. J. Cell Biol. 134:793-799, 1996.

V. APPENDIX

1. Figure Legends and Figures

Figure 1. *Southern blot of tail DNA from WAP-ST-3 transgenic mice.* Tail DNA (10 μg) from 8 founders (lanes 1-8) and 1 control (lane 9) were digested with Sac I, fractionated on a 1% agarose gel, blotted and the membrane hybridized to [^{32}P]-labeled human ST-3 probe. The 8 transgenic founders were identified from a total of 12 candidate mice.

Figure 2. *ST-3 mRNA expression in transgenic mouse mammary gland.* Whole mammary gland RNA (10 μg) from 4 week old virgin (lane 1), 10 week old virgin (lane 2), 16 day pregnant (lane 3), 2 day lactating (lane 4), 5 day lactating (lane 5), 10 day lactating (lane 6) and 3 day involuting (lane 7) P3 transgenic mice was separated on a 1% agarose gel in the presence of formaldehyde, transferred to a nylon membrane and hybridized to [^{32}P]-labeled probe for human ST-3. Normal mouse mammary glands at the stages presented do not express ST-3 until day 3 of involution wherein the signal is ~50 times lower (data not shown).

Figure 3. Hematoxylin-eosin staining of 4 week old virgin glands from normal (A) and P1 (B), P3 (C) or P9 (D) transgenic animals.

Figure 4. Hematoxylin-eosin staining of 16 day pregnant glands from normal (A) and P1 (B), P3 (C) or P9 (D) transgenic mice.

Figure 5. Hematoxylin-eosin staining of 5 day lactating glands from normal (A) or P1 (B), P3 (C) and P9 (D) transgenic mice. Note collapse of alveoli in P1 transgenics and the decrease in size of alveoli as well as the mass of alveolar structures in P3 and P9 transgenics.

Figure 6. *Staining for apoptotic cells and TEM analysis of 16 day old pregnant glands.* Mammary glands from normal (A,B) or P3 transgenics (C,D) were removed following 16 days of pregnancy and stained for apoptotic cells by the ApopTag method (nuclei in apoptotic cells stain dark and are marked by arrows; A and B). Basement membrane integrity was examined by TEM. In B, THE intact basement membrane is indicated by arrow while in D, a portion of the disrupted basement membrane is indicated by the cross.

Figure 7. *TEM analysis of mammary gland after 5 days of lactation.* In panels A and B, glands from normal animals show normal alveoli with intact basement membranes. In contrast, glands from lactating transgenic animals (C and D) show marked increase in apoptotic cells (indicated by arrows) as well as changes in basement membrane structure beneath apoptotic cells (arrow in panel D).

Figure 8. Hematoxylin-eosin staining of glands from normal (A) and P1 (B), P3 (C) or P9 (D) transgenic mice 3 days post-weaning.

Figure 9. A and B) A 1.5 mm^2 tissue fragment isolated from murine abdominal wall muscle was embedded in fibrin/collagen matrix, as described, and cultured for 0 or 18 days. Phase microscopy (x120) demonstrates that numerous vessels can be observed after *in vitro* culture for 18 days (panel B; arrowheads). Because of the three-dimensional nature of the gel, only a portion of the anastomosing network can be seen. C and D) Light (x300) and electron (x4350) micrographs of an 18-day old culture reveals numerous patent lumens (marked "L") comprised of a single layer of endothelial cells. A myofibroblast-like cell can be seen in juxtaposition with the vessel near the bottom of panel D. E and F) Transmission electron micrographs of patent vessels

(18-day old culture) demonstrating tight junctions and a deposited basement membrane (both indicated by arrowheads) as shown in panels E (x42,000) and F (x53,000), respectively.

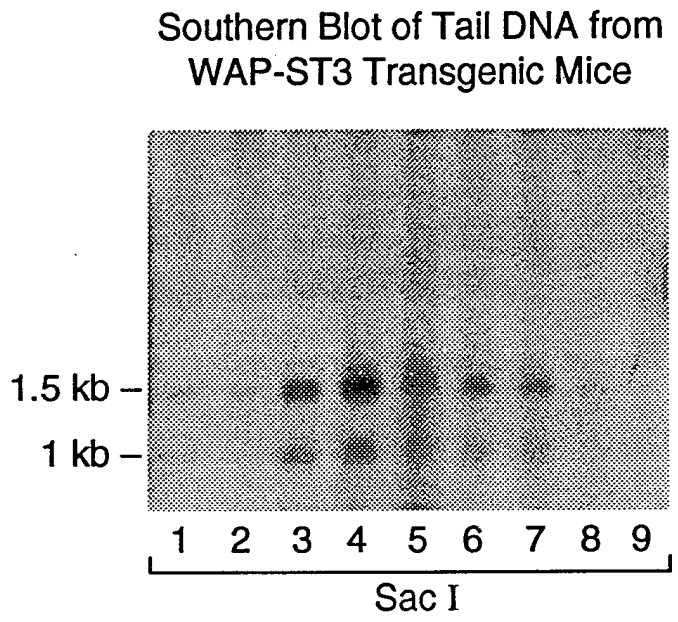
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Figure 1

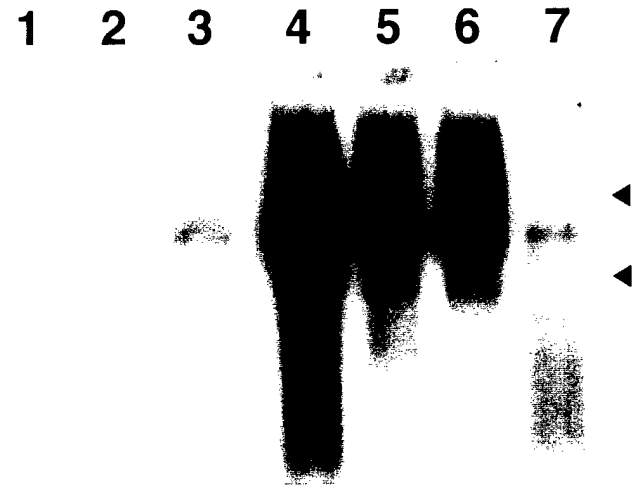

Figure 2

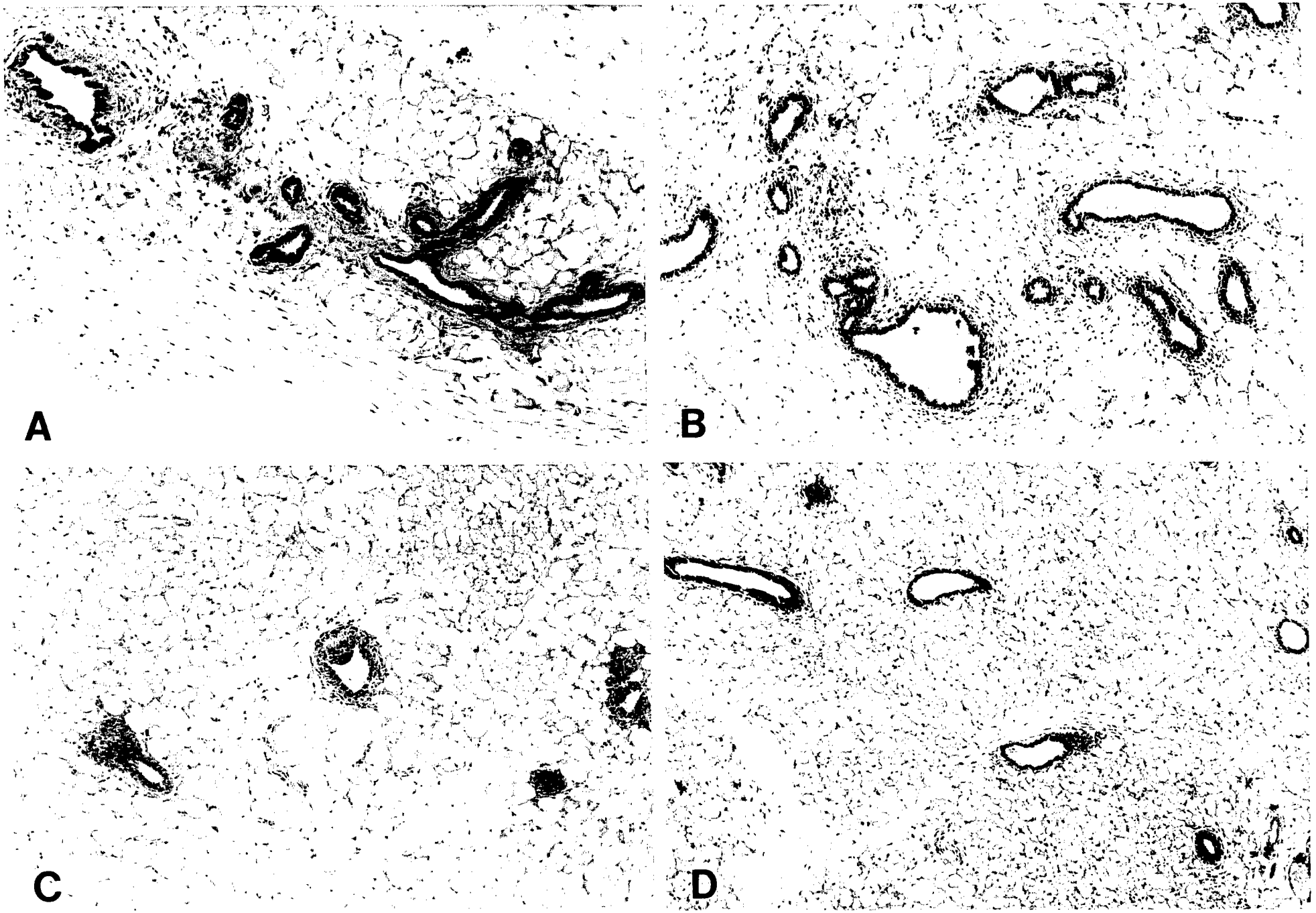

Figure 3

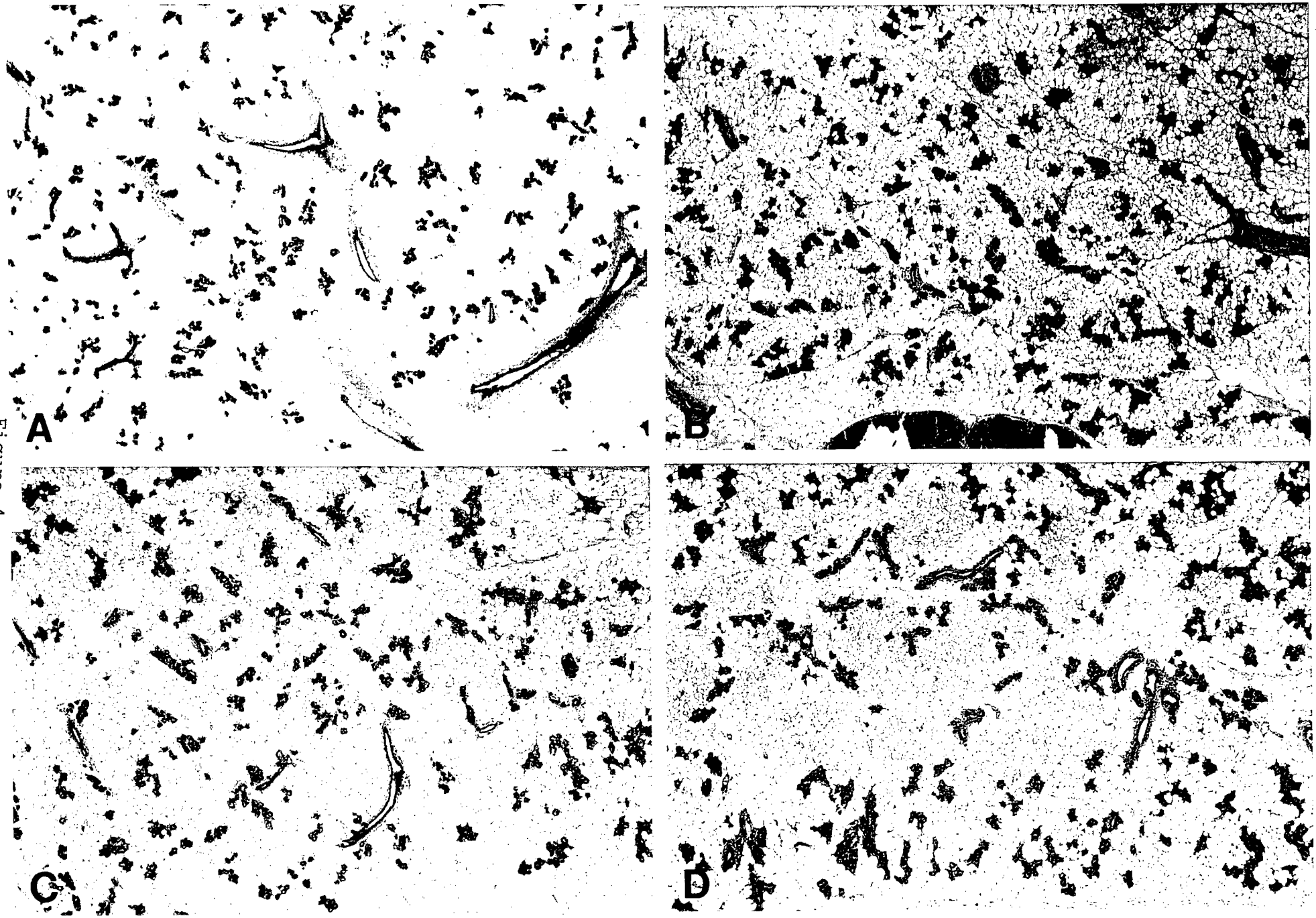

Figure 4

16 day pregnant

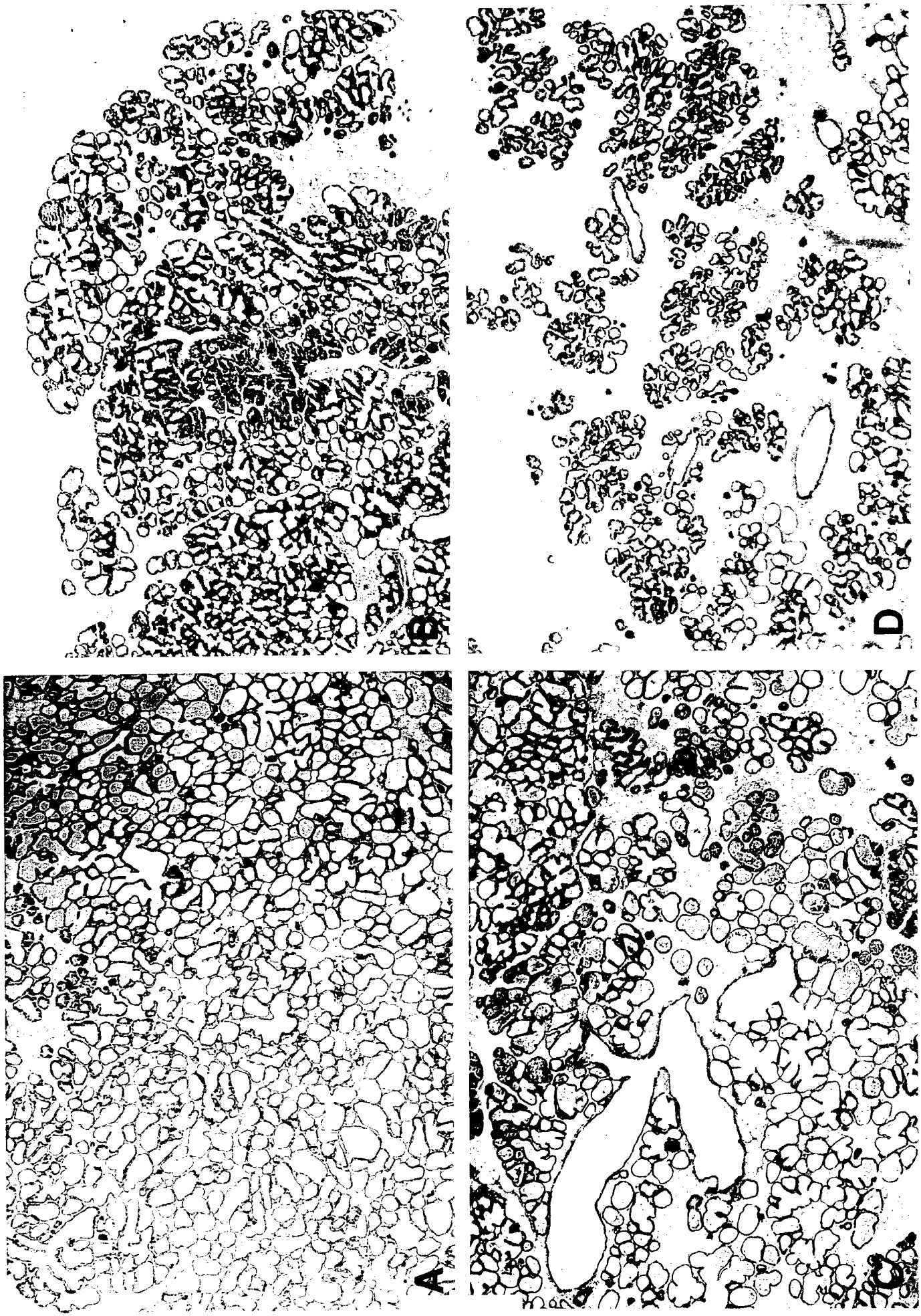

Figure 5

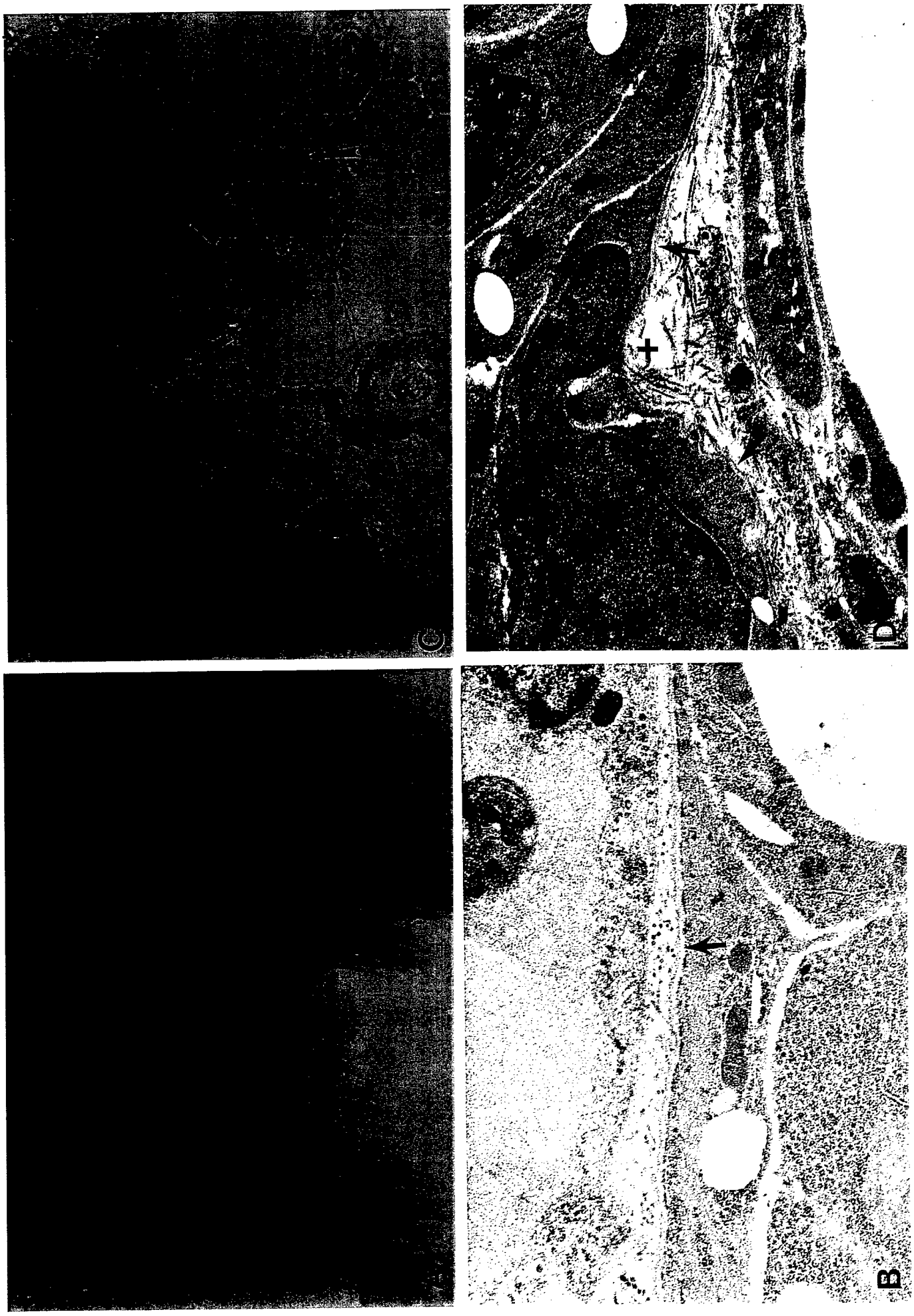

Figure 6

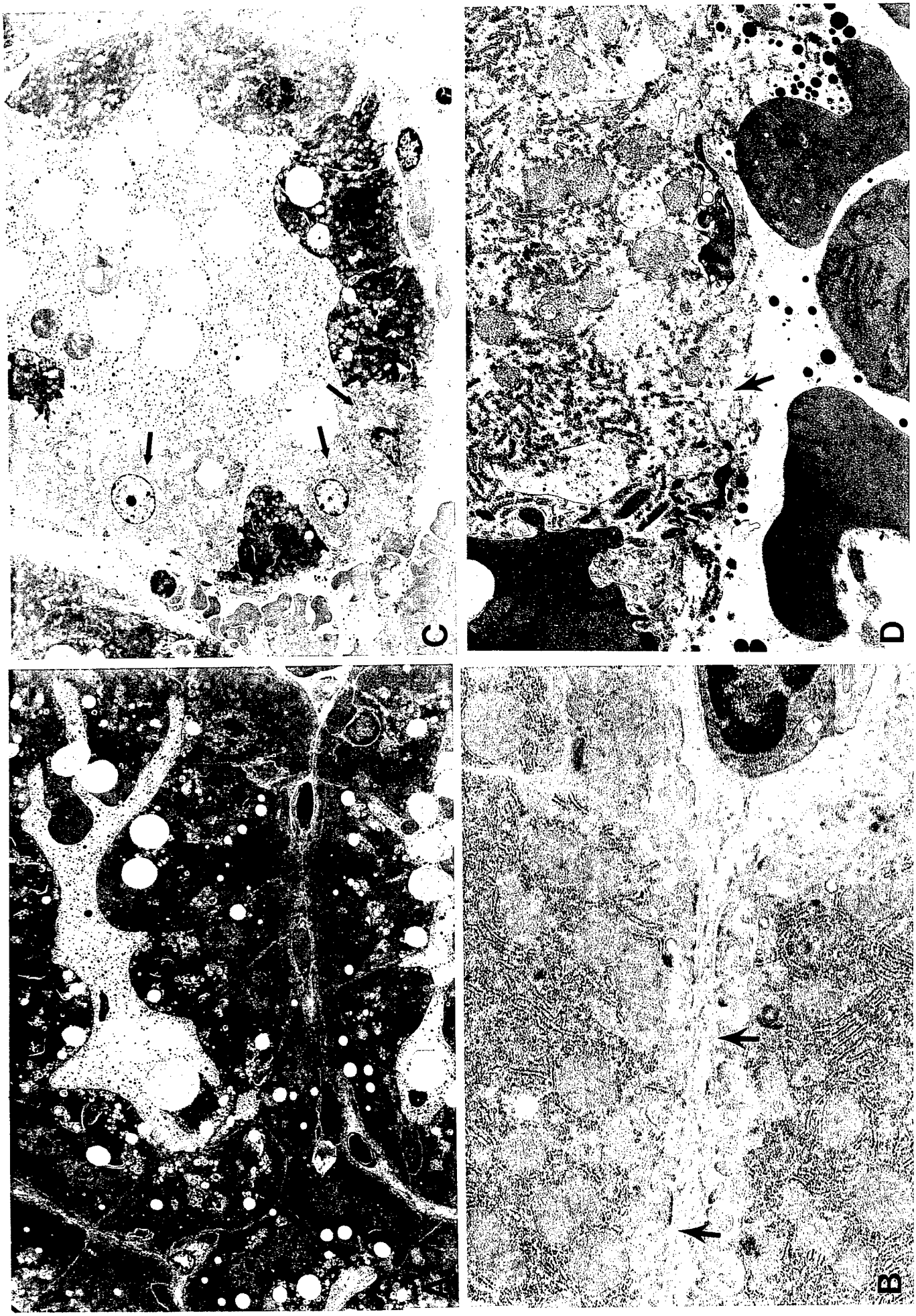

Figure 7

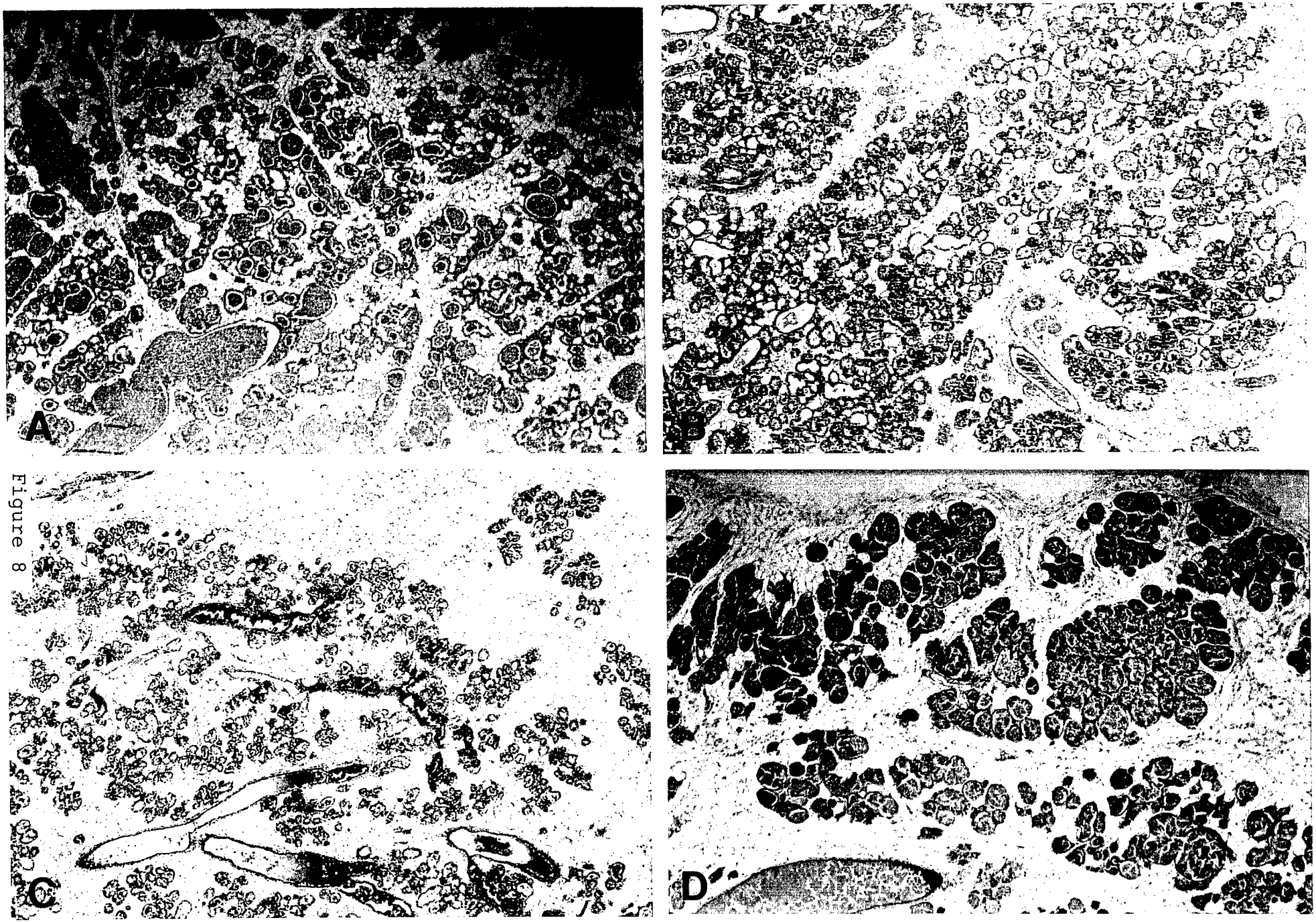

Figure 8

3 day involuting

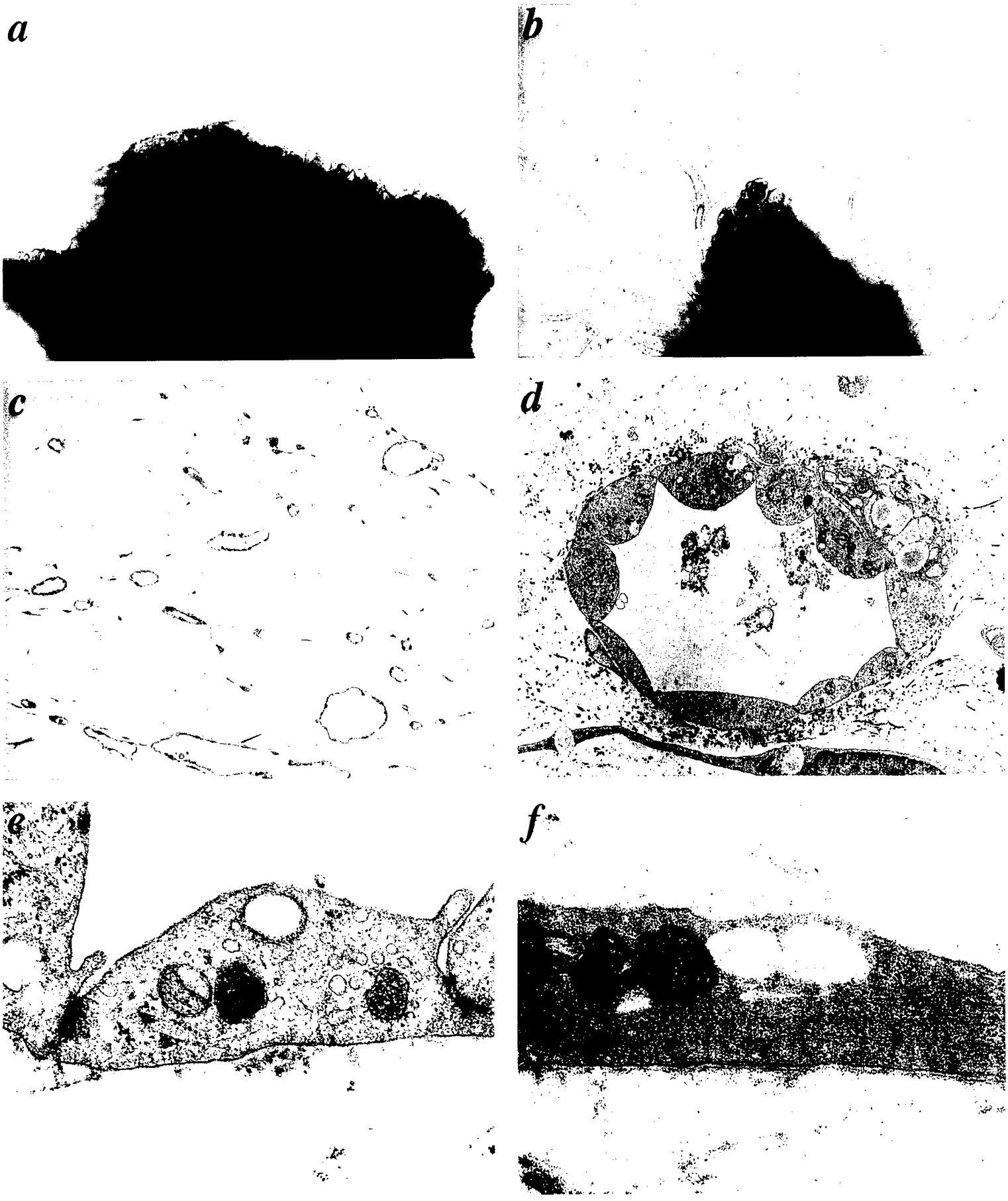

Figure 9